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(54) Title: PORCINE ANTIBODIES TO TNF-α(ALPHA)

(57) Abstract

Porcine anti-TNF-α (cachectin) antiserum, antibodies, and methods for obtaining such are disclosed. The invention teaches the production, screening, purification, and testing of porcine anti-human-TNF-α antibodies. The methods and antibodies of the present invention are useful for pharmaceutical, clinical, laboratory and diagnostic uses.

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TITLE: Porcine Antibodies to TNF-α (alpha)

FIELD OF THE INVENTION

This invention relates the production of polyclonal porcine antibodies to human TNF-α (cachectin) and the uses of the polyclonal antibodies thereof.

BACKGROUND OF THE INVENTION

Cytokines are a broad class of naturally occurring peptide which are released by cells as extra-cellular signals. Inflammatory cytokines are produced at the sites of inflammation by infiltrating mononuclear cells of the immune system. These cytokines play a beneficial role as immunostimulants and mediators of other responses to infectious agents. Tumor Necrosis Factors are a family of two proteins, TNF-α (cachectin) and TNF-β (lymphotoxin). TNF-α is produced by monocytes and macrophages (Beutler, B. et al., Nature 316:552 (1985)) and has also been reported to be produced by Natural Killer (NK) cells (Cuturi, M., et al., J. Exp. Med. **165**: 1581 (1987)). Certain human tumor cells have been reported to produce minute amounts of TNF-α spontaneously (Cordingley, F.T., et al., Lancet **I**: 969 (1988)).

TNF-α (cachectin) was originally hailed as a selective anti-tumor agent, it is now thought that the overproduction of TNF-α (cachectin) during infection leads to severe systemic toxicity and even death (Vilcek and Lee, J. Biol. Chem. **266**: 7313-7316 (1991)). TNF-α (cachectin) has been implicated in the development of septic shock after Gram-negative bacterial infection (Tracey, K.J. et al., Nature **330**: 662 (1987)), pathogenesis of auto-immune disease (Pujol-Borell, R., et al., Nature **326**: 304 (1987)), and graft-versus host disease (Piguet P.-F., et al., J. Exp. Med. **166**: 1280 (1987)). Elevated levels of TNF-α (cachectin) have been found in all stages of HIV infections and in AIDS (von Sydow, M., AIDS Res. and Human Retroviruses, **7**: 375 (1991)). Thus TNF-α (cachectin) is implicated as an element associated with severe reaction to a variety of disease states.

TNF-α (cachectin) can affect a variety of cells which have receptors for TNF-α (cachectin). B-lymphocyte proliferation is inhibited by exposure (Kashiwa, H. et al., J. Immunol. 138: 1383 (1987)). Response to TNF-α (cachectin) can result in suppression of lipoprotein lipase, carboxylase and fatty acid synthetase thereby modulating the metabolic activities of adipocytes (Torti, F.M. et al., Science 229: 867 (1985)). TNF-α (cachectin) lowers the membrane potential of isolated skeletal muscle cell preparations, and may also be involved in the breakdown of muscle proteins (Goldberg, A. et al., J. Clin. Inves. 81: 1378 (1988)). The shock promoting effect of the hemorrhagic necrosis brought on by exposure to TNF-α (cachectin) appears to be caused by the down-regulation of endothelial cell expression of thrombomodulin, thereby causing procoagulant activity (Stern, D.M. et al., J. Exp. Med. 163: 740 (1986)).

TNF-α (cachectin) also has a variety of effects on the hematopoetic system. TNF-α (cachectin) enhances the adhesion of neutrophils to endothelial cells, promotes the release of lysozyme by them, and their degranulation (Shalaby, M.R. et al., J. Immunol. 135: 2069 (1985)). TNF-α (cachectin) has a toxic effect on the liver by depressing various important enzyme systems involved in metabolism, and as a consequence, hepatic protein synthesis is suppressed (Gaskill, H.V., J. Sug. Res. 44: 664 (1988)).

Experiments with TNF-α (cachectin) on animals have helped to illuminate the mode of action of the toxic events triggered by large doses of TNF-α (cachectin). Approximately 10% of a dose of TNF-α (cachectin) injected i.v. will become localized in the gastrointestinal tract (GI tract) leading to bowel necrosis. This triggers the release of lipopolysaccharides (LPS) from the bowel which leads to irreversible endotoxin shock (Tracey, K.J. et al., Science 234: 470 (1986); Patton, J.S. et al., J. Clin. Invest. 80: 1587 (1987)). Anorexia can be caused by TNF-α (cachectin) acting on the cerebral hypothalamus region directly. Pyrogenic effects may also be partly or entirely derived from action on this region (Plata-Slman, C.R. et al., Brain Res. 448: 106 (1988)).

The use of TNF-α (cachectin) as an anti-tumor agent has yielded mixed results. In a Southwest Oncology Group (SWOG) Study Protocol, recombinant TNF-α (cachectin) was administered as an intravenous infusion in patients with advanced adenocarcinomas at doses starting at 160 ug/sqm per day in order to study the antitumor effects. There were numerous toxic side effects associated with this treatment. These included fever, rigors, nausea, vomiting, anorexia, flu-like symptoms, hypotension, hyperglycemia, anemia, coagulopathy, hepatotoxicity, and hpertriglyceridemia. There was laboratory evidence of disseminated intravascular coagulopathy (DIC) in 50% of the patients; two patients developed pulmonary emboli. No objective response on the patient's tumors was observed (Brown, T.D. et al., J. Immunotherapy **10**: 376 (1991)). These toxic side effects preclude the use of TNF-α (cachectin) as an anti-tumor agent.

TNF-α (cachectin) consists of 157 amino acids, and the genes have been cloned. Under denaturing conditions, the molecular weight of TNF-α (cachectin) on SDS-PAGE gel was found to be 17,000 daltons . There is also evidence that there is a longer membrane anchored precursor form (Vilcek and Lee, J. Biol. Chem. **266**: 7313 (1991)).

Certain HIV-1 envelope peptides derived from both gp41 and gp160 have proven to induce increased levels of gp41 and it has been described that TNF-α has the capacity to reactivate HIV infection via trans-activating mechanisms in monocytes and macrophages (von Sydow, M. et al., AIDS Research and Human Retroviruses, **7**: 375 (1991)). If TNF-α is found to up-regulate HIV-1 and interferon alpha was found to down-regulate HIV-1, a vicious circle resulting in an eventual exhaustion of interferon producing cells, together with a failing efficacy of the cell-mediated and humoral immune system, will potentiate the release and effect of TNF-α .

High levels of TNF-α in advanced HIV-1 infected patients may contribute considerably to their deteriorating clinical condition and may play a major role in AIDS-related symptoms such as fatigue, weight loss, fever and anorexia. Furthermore, by its multiple effects on various organs, among them the bone marrow and the immune

system, may participate in exacerbating immune deficiency and at the same time up-regulation of HIV-1 proliferation. Interferon alpha levels also appear elevated in HIV-1 infected patients, and this may be due to an anti-viral function of interferon alpha. Experiments in vivo with un-infected individuals with interferon alpha levels similar to HIV-1 infected individuals showed that there was a marked anti-viral effect (von Sydow, *supra*).

TNF-α is most likely a more potent HIV-1 stimulator than Interferon alpha is as an HIV-1 inhibitor, and both may function antagonistically. Attempts to treat patients with recombinant interferon alpha has failed to improve the clinical condition of HIV-1 infected individuals.

SUMMARY OF THE INVENTION

The present invention demonstrates the production of antibodies to human TNF-α (cachectin) from a porcine source. These antibodies can be polyclonal, or made monoclonal and are preferrably of a porcine origin.

The antibodies of the present invention are useful for the labeling of cells that may display the TNF-α (cachectin) molecule, or have it bound to surface receptors; in assays and assay kits for the determination of the amount of TNF-α (cachectin) in bodily fluids or tissue samples. An advantage with use of polyclonal serum containing polyclonal porcine antibodies to TNF-α (cachectin) is that a greater range of affinities for the TNF-α (cachectin) would be present, and therefore allow for a functionally higher affinity of detection.

Antibodies of the invention can be used to counteract the toxic effects of TNF-α (cachectin) in systems where TNF-α (cachectin) is being administered, in vitro or in vivo. By administering the antibodies of the invention in conjunction with TNF-α (cachectin), systemic, non-specific, and un-desired reactions can be mitigated. In patients determined to have elevated levels of TNF-α (cachectin), a quantitative amount of the antibodies of the invention can be administered to neutralize the TNF-α

(cachectin). An advantage of the antibodies of the present invention is that even in patients sensitized to murine or other species of antibodies, porcine antibodies of the invention can be an effective treatment avoiding many of the immunotolerance problems associated with using antibodies generated in other species. The antibodies of the present invention, made by the process of the present invention, would thus be an effective therapy when patients have become unable to tolerate treatment by antibodies generated in other species of animals.

The antibodies of the invention are also useful for the generation of anti-idiotype antibodies that can mimic the action of TNF-α (cachectin), and provide an alternative protein for the use as an anti-tumor agent.

The antibodies of the present invention, generated by the methods taught in the present specification can be digested to form antigen-binding fragments which are missing major non-binding regions of the protein. Such modified antibodies are possibly even less immunogenic, than the whole antibody would be. Such manipulation and construction of recombinant porcine antibodies are all made possible by the methods of the present invention, which teach the generation of porcine antibodies to human TNF-α (cachectin), the identification, and purification of the antibodies produced thereby.

In a prefered embodiment of the instant invention, the invention is a polyclonal porcine antiserum which binds to human TNF-α (cachectin). This antiserum is the product of a process which is a method for the production of such antibodies comprising the steps of

a) attaching human TNF-α (cachectin) to a suitable carrier molecule,

b) emulsifying the TNF-α (cachectin) and suitable carrier in an appropriate adjuvant,

c) injecting the TNF-α (cachectin), carrier and adjuvant into pigs,

d) and isolating and testing the serum from pigs for anti-TNF-α (cachectin) activity.

In a preferred embodiement, the porcine polyclonal serum produced by the method described above is a polyclonal anti-serum containing polyclonal antibodies that will inhibit the binding of anti-TNF-α (cachectin) antibodies produced in other species.

In another prefered embodiemnt, the polyclonal serum generated as in above can be used to produce monoclonal antibodies reactive to TNF-α (cachectin) which inhibits the binding of the polyclonal antibodies to TNF-α (cachectin).

The present invention also has as a prefered embodiment, a method for inhibiting the activity of TNF-α on cells comprising the steps of administering to the cells an effective amount of anti-TNF-α antibodies as described above to the cells.

In a preferred embodiment, the antibodies of the present invention are used to mitigate the effects of TNF-α (cachectin) in mammals. Most preferably humans. In the preferred embodiment, the polyclonal antibodies are administered to the subject patient via injection, infusion, bolus, orally, or by implantation. The most preferred being injection or infusion as a bolus or as a pharmaceutical composition in a suitable solvent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a photograph of a Western Blot using the antibodies of the invention demonstrating the binding to, and detection of TNF-α (cachectin). A TNF-α specific protein band was detected at 17,000 Daltons. As estimated from the Western blots the anti TNF-α antibody response produced in pig #11 was stronger than that found in pig #10. There was no reaction between serum from the non-immunized pig. The various lanes are dated as to the test bleed for each pig on the date the blood was drawn. The molecular weight is indicated by the scale on the side.

Figure 1b is a photograph of a Western Blot analysis of pig #11, comparing pre-bleed (pre-immune) sera with hyperimmune sera at various dilutions. (A - hyperimmune sera, diluted l-r. 1,000; 3,000; 6,000; and 12,000; B - pre-immune sera).

Figure 2 is a photograph of a microtiter plate showing the assay of Example 4.

DETAILED DESCRIPTION OF THE INVENTION

The present invention contemplates and demonstrates the production of antibodies to human TNF-α (cachectin) from a porcine source. The antibody products of the process taught by the present invention are useful for diagnostic, therapeutic, clinical as well as basic research applications. The antibodies of the present invention can be polyclonal, or made monoclonal. The composition of a polyclonal antiserum can consist of many sub-classes of immunoglobulin molecule, and may consist of IgM, IgG, IgE, IgD, and IgA. In the preferred embodiment, the predominant subclass is IgG. A polyclonal antiserum is characterized by a heterogeneous collection of binding specificities, with the common thread being the ability to bind to the target protein, in this case TNF-α (cachectin). A polyclonal serum can serve as the basis for further purifying from that sera, specific antibodies of higher and higher affinity for the target protein. The use of a porcine system allows for the flexible production of antiserum, that would be a greater source of serum when compared with small rodents. The porcine response maybe in qualitative measure, better than the response possible in other more traditional systems. Antibodies which could bind to the TNF-α (cachectin) molecule generated from a porcine source would be useful for in vivo therapeutics for patients sensitized to murine or other types of antibodies and for in vitro testing. Such antibodies could be used in assays, and assay kits for the determination of the amount of TNF-α (cachectin) in bodily fluids or tissue samples.

The methods of conjugation of TNF-α to a carrier molecule, preferably uses KLH (key-hole limpet hemacyanin) and a cross-linking reagent to react the sulfhydrl groups on TNF-α (cachectin) to KLH. A maleimide group on the surface of KLH reacts with sulfhydrl groups on TNF-α (cachectin) to form a stable thioether bond. One with ordinary skill in the art will realize that there can be several similar methods for accomplishing the same result.

The monoclonal antibodies (mAbs) of the present invention can be produced by antibody producing cell lines commonly referred to as hybridomas. The preferred monoclonal antibodies of this invention are reactive with TNF-α (cachectin) antigens. The hybridomas are formed by the fusion of the antibody producing cell and an immortalizing cell line; that is, a cell line which imparts long term tissue culture stability on the hybrid cell. In the formation of the hybrid cell lines, the first fusion partner (the antibody producing cell) may be a spleen cell of an animal (preferably a pig) immunized against TNF-α (cachectin). The second fusion partner (the immortal cell) may be a lymphoblastoid cell or a plasmacytoma cell such as a myeloma cell, itself an antibody producing cell but also malignant. The preferred fusion partner is a porcine cell, or a cell from a human or other primate. Fusions can be accomplished using standard procedures (Kohler and Milstein, (1975) Nature 256:495-97; Kennet, R. (1980) in *Monoclonal Antibodies*, Kennet et al., Eds. pp 365-367, Plenum Press, N.Y.; Buchegger F., et al., JNCI 79:337-342 (1987)).

The hybridomas are screened for production of antibody reactive with TNF-α (cachectin) antigens, and those which secrete reactive antibodies are cloned. The desired monoclonal antibodies can be recovered from the hybridoma supernatant using conventional techniques.

The polyclonal and monoclonal antibodies (mAbs) of the present invention can be used, for example, in diagnostic kits for as antibodies in ELISA, RIA, SPRIA, Chromatographic, Bead-linked, Magnetically-isolated, Chemiluminesent, Fluorescent antigen detection systems, for direct visualization or cell and tissue labeling, in treatment of cell cultures, or in the treatment of humans. Therapies and uses contemplated with polyclonal antiserum to TNF-α (cachectin) allow that monoclonal antibodies could be used in their place. Such therapies encompass the use of TNF-α antibodies to mediate the effects of TNF-α in mammals, whereby the cells and tissues could be protected by the antibodies of the instant invention.

Those skilled in the art will appreciate that naturally occurring TNF-α (cachectin) antigens, fragments thereof, recombinant products or synthetic peptides can be used, for example, to stimulate antibody producing cells following the teaching of the present invention.

Those with ordinary skill in the art will recognize that the teachings of the instant specification enable the production of antibody fragments with equivalent binding as the whole antibodies of the present invention, and are thus taught by the instant specification. The development of chimeric antibodies, humanized antibodies, single-chain antigen binding proteins, and other manipulations known in the art, that bind to TNF-α , would only be using the teachings and antibodies of the present invention. The antibody products of the process taught by the present invention are thus inclusive of the nature of the binding active site and the immunoreactive sites of the molecule.

By taking a biopsy of spleen tissue from the live pigs, monoclonal antibodies can be generated that correspond to the antibodies in the polyclonal response. By keeping the pigs alive, the source of spleen tissue remains viable. As a means for the generation of antibodies to proteins that may not illicit a response in traditional systems, the porcine system can serve as a unique immunoglobulin gene source.

The following examples are meant by way of illustration and are not intended as a limitation of the disclosure contained here of the instant invention. Those who are skilled in the art will know, or will be able to derive, using no more than routine experimentation, any equivalents to the specific embodiments of the present invention.

Example 1

Production of TNF-α (cachectin) Immunogen

TNF-α (cachectin) was conjugated to Keyhole Limpet Hemocyanin (KLH) by using a cross-linking reagent to react the sulfhydrl groups on TNF-α (cachectin) to KLH. A maleimide group on the surface of KLH reacts with sulfhydrl groups on TNF-α

(cachectin) to form a stable thioether bond. Maleimide activated KLH was obtained from Pierce (Illinois).

Although TNF-α (cachectin) contains two cysteine residues, a primary study revealed that they are not accessible for conjugation. Therefore, additional SH (sulfhydrl groups) were introduced into the TNF-α (cachectin) using SPDP (N-succinimidyl-3-(2-Pyridyldithio)propionate).

TNF-α (cachectin) was dissolved in phosphate buffered saline (PBS) (pH7.2-7.4) at a concentration of 0.5 mg/ml and incubated with 15 fold excess of SPDP dissolved in DMSO for two hours. A 2-pyridyldisulfide group was introduced into TNF-α (cachectin) when SPDP reacted with the primary amine (or amino groups) of the protein. The pyridine-2-thione groups were then released by incubation with 4 mM dithiothreitol (DTT) for 30 minutes at room temperature. The unreacted SPDP and its reduction products were then removed using a desalting column equilibrated in PBS. After desalting the thiolated TNF-α (cachectin) was ready for the conjugation step.

Thiolated TNF-α (cachectin) (0.25 mg) was prepared as described above, in 1 ml of PBS and added to 2 mg of maleimide activated KLH in 0.1 ml of 83 mM phosphate buffer (containing 0.9 M NaCl and 0.1 M EDTA, pH 7.2). The mixture was stirred for four hours at room temperature to allow the formation of a stable thioether bond between the sulfhydryl group on the TNF-α (cachectin) and the maleimide group on the KLH.

Example 2

Immunization of Pigs and Purification of Porcine Antibodies

Two pigs (mixed Yorkshire breed, approximately 60 lbs.) were immunized with 1 mg of the TNF-α -KLH conjugate prepared as described in Example 1. The conjugate was emulsified in Freund's Complete Adjuvant. The pigs were immunized by subcutaneous immunization on Day 0. This was followed by a booster immunization consisting of the conjugate emulsified in Incomplete Freund's Adjuvant on Days 1, 21,

42 and 63. Injection schedules and composition formulations useful for immunization are well known to those skilled in the art. Test bleeds were drawn from the pig's ear veins 7 days after each injection. The presence of antibodies to the antigen can, most conveniently be detected by the Western Blot technique. The antibody titer can be analyzed by standard Enzyme Linked Immunoabsorbent Assay (ELISA) techniques.

The immune porcine IgG may be purified by precipitation with polyethylene glycol (e.g. PEG 8000) prior to use. A suitable polyethylene glycol precipitation technique has been described by Carter and Boyd (J. Immunol. Meth. 26:213 (1979)). This purification method eliminates any hemolysis from the blood and removes the majority of alpha and beta globulins. IgG products having concentrations of from about 4.5%-5.5% w/v may thus be provided, analogous to human polyethylene glycol-precipitated immunoglobulin such as "Immunoglobulin 7S Human IV," marketed by Armour Pharma of Germany. This form of immunoglobulin may be intravenously administered (see, for example, Stanley, P. and Cole, P., Lancet, I:829 (1983)). A preferred purification method is by ion exchange chromatography or by immunopurification methods. Ion exchange chromatography is well known to those of skill in the art and exploits the charged nature of a molecule at a given pH to effect separation. Immunopurification methods involve the use of a moiety which specifically binds to the immunoglobulin in an immunoreactivity-based purification scheme. For example, it is known that protein A from *S. aureus* binds specifically to IgG. Protein G from *streptococci*, for example, is also useful for this purpose. Thus, a purification scheme can be designed wherein protein A is fixed to a solid support such as a column packing material. Porcine serum can be passed through the column and the IgG component is retained in the column and other serum components pass with the flow-through fraction.

Example 3

Western Blot Characterization of Porcine Antibody Activity

Approximately 10 pg of purified TNF-α (cachectin) was electrophoresed on a 17.5% SDS-PAGE gel using the procedure described by Laemli et al. (REF). The gel containing the electrophoesed TNF-α (cachectin) was then electro-transfer blotted onto nitrocellulose paper. The non-occupied binding sites of the nitrocellulose were then blocked by using 5% non-fat milk/0.1% - Tween 20 in PBS. The nitrocellulose paper containing the blotted protein was then cut into strips.

Serum from the immunized pigs were then tested for the presence of anti-TNF-α (cachectin) antibodies. The TNF-α (cachectin) Western Blot strips, prepared as above, were incubated with the serum from the test pigs #10 and #11, as well as with serum from a control non-immunized pig, #12. The serum was diluted 1:100 in PBS containing 5% non-fat milk and 0.1% Tween 20. The diluted serum was added to each strip (3 ml) and incubated for 1 hour at room temperature and washed 3 times with PBS Tween 20 buffer. The anti-TNF-α (cachectin) antibodies bound to the TNF-α (cachectin) attached to the strips were visualized by adding goat-anti-pig IgG (heavy and light chain) conjugated to alkaline phosphatase. The color reaction was produced after reacting the bound conjugated detection antibody with BCIP (5-bromo-4-chloro-3-indoylphosphate p-toluidine salt) and NBT (p-nitrobluetetrazolium chloride mixed substrate (Bio Rad, California). Molecular markers on each strip were stained with Coomassie blue.

Figure 1a illustrates the results from such a Western Blot. TNF-α (cachectin) specific protein bands were detected at 17,000 daltons (monomer form of TNF-α), 34,000 daltons (dimer form), 51,000 daltons (trimer form) and 68,000 daltons (tetramer). As estimated from the Western blots, the anti-TNF-α (cachectin) antibody response produced by pig #11 was stronger than that found in pig #10. There was no reaction detected from pig #12, the negative control.

Figure 1b compares pre-immune and dilutions of hyperimmune sera from pig #11.

Example 4

TNF-α (cachectin) Neutralization Assay

TNF-α exhibits a cytolytic effect on several transformed cell lines *in vitro*. These cell lines bear TNF-α receptors on their surface, the binding of which by TNF-α leads to cell lysis. Thus determination of cell lysis of TNF-α sensitive cells *in vitro* serves as a predictive model system which is utilized to quantitatively measure TNF-α activity. This model system also becomes useful for the determination of inhibitors of TNF-α activity as well. The antibodies of the instant invention were tested using this system.

Briefly porcine seurm samples from the pigs immunized with TNF-α (cachectin)-conjugate as described above, and tested by TNF-α (cachectin) Western Blot analysis as described above were serially diluted 1:2 in 96 well microtiter plates containing 25 ul cell culture medium (MEM plus 10% fetal calf serum) per well. The plates were incubated for 1 hour at 37°C, following which $2x10^4$ L929 cells in 100 ul MEM plus 10% fetal calf serum were added to each well. The plates were then incubated for an additional three days at 37°C, in a 5% CO_2 incubator. The wells were then visually inspected for neutralization of cytotoxicity on an inverted microscope.

Methods

L929 cells were plated on 96-well flat-bottomed microtiter plates, and incubated at 37°C and allow to grow until a monolayer was formed, before addition of KLH-TNF-α and porcine anti-TNF-α hyperimmune antisera. The anti-TNF-α sera was serially diluted 2-fold in culture medium from 1:40 to 1:20,480. An equal volume of 100 units/ml TNF-α in the same culture medium was added to the diluted porcine sera. After incubation for 30 minutes at 37°C to allow for the anti-TNF-α antibodies to react with the TNF-α , 50 µL of the antibody/TNF-α mixture was transferred to each well containing a monolayer of cells in the microtiter plate. The plate was then incubated at 37°C overnight in order for cell lysis to occur. The detatched lysed cells were removed from the wells by washing, and the remaining viable adherent cells were measured by

staining with crystal violet. The higher the color intensity of the crystal violet stain in each well, the more live cells remaining.

Results

The color intensity after staining for control wells showing 100% cell viability is shown in Figure 2, as rows A and H, and in column 1 and 12 of the microtiter plate. The control wells were not treated with TNF-α.

In contrast to the control wells, wells in rows B and C show 100% cell death, leaving no detectable viable cells. In these wells, cells were exposed to mixtures of TNF-α and pre-immune porcine sera (pig 11), treated in the same fashion as the hyperimmune sera. TNF-α in these samples were fully active after incubation with the pre-immune sera.

Wells in rows D, E, F, and G reflect the neutralization of TNF-α by incubation with anti-TNF-α hyperimmune sera of the instant invention. The killing effect of TNF-α was greatly inhibited by the treatment (D and E, pig 11; F and G, pig 31). Columns 10 and 11 contained the most highly diluted hyperimmune sera. The ability of porcine anti-TNF-α hyperimmune sera to neutralize TNF-α began to decrease at dilutions greater than from about 1:5000 to 1:10,000 (columns 9 and 10). The results show that the hyperimmune sera contains active titers of the antibodies of the instant invention that is effective in neutralizing TNF-α activity on living cells.

Antibody titers were calculated by multiplying the dilution of serum that provided protection against 40 units/ml of TNF-α in 50% of the cells (effected LD_{50}) times 40 (adjustment for per unit/ml). Therefore, a well containing a 1:100 dilution of TNF-α serum, in which 50% of the cells were still viable after incubation, would yield a antibody titer of 100 x 40 = 4000 neutralizing units/ml (NTU/ml). The use of this assay showed that the porcine serum collected one week after the sixth injection with recombinant TNF-α - conjugate had a titer of consistently greater than 100,000 NTU/ml. This titter would be appropriate for harvesting polyclonal porcine anti-TNF-α antibodies from the serum. Elevated TNF-α levels are higher than 6 ng/ml in the

circulating blood, and the specific activity of TNF-α is understood to be about 10^{-7} mg for 1 Unit of activity. Therefore an effective amount of anti-TNF-α antibodies can be obtained using the method of the present invention.

Example 5

Immunization with Unconjugated TNF-α (cachectin)

Serum draw from 24 pigs immunized 5 times with KLH-TNF-α (in Complete Freund's adjuvant first two times, incomplete Freund's the rest) showed consistent results using the assay of Example 4, of around 1 x 10^6 NTU/mL.

Serum drawn form two pigs immunized with unconjugated recombinant TNF-α (50 μg per immunization) 5 times using the same schedule as for KLH-TNF-α, as above, also showed 1 x 10^6 NTU/mL. Thus, suprisingly, it is evident that effective immunization can be accomplished with unconjugated as well as conjugated TNF-α.

Example 6

Preparation of Monoclonal Antibodies

The spleen of the immunized pig can be removed aseptically within minutes of death and cooled on ice. Alternatively, a sterile biopsy can remove spleen cells without killing the animal, and also cooled on ice. The specimen (about 5-8 g) of tissue can be washed in serum-free RPMI medium (Biochrom KG, Germany), minced, and homogenized. Batches of 5 x 10^8 spleen cells can then be fused with a 5 to 10 times lower number of myeloma cells from the P3-NSI/1-Ag4-1 line (or other suitable fusion partner) with the use of 1.5 ml polyethyleneglycol 2000 at 40% (wt/vol) in serum-free medium. After being washed, the cells of the fusions can be dispersed in 96-well plates containing RPMI medium supplemented with L-glutamine (0.3 mg/ml) and 10% fetal calf serum and mouse peritoneal macrophages. Hypoxanthine-aminopterin-thymidine selective medium can then be added to the cultured cells from days 1 to 21 after the fusion.

For screening of anti-TNF-α (cachectin)-producing hybridomas, a sample of culture medium from growing hybridoma cells can be incubated with purified TNF-α (cachectin) that has been radiolabelled with ^{125}I by the chloroamine-T method for RIA detection after ammonium sulfate precipitation of the antibodies, or incubated with immobilized TNF-α (cachectin) for ELISA detection. Hybridoma specimens which repeatedly bind to TNF-α (cachectin) can be selectively cloned in 96-well culture plates.

The identification of the monoclonal antibody species and isotype can be accomplished using various EIA. For example, to detect the production of pig IgG, the hybridoma culture supernatants can be incubated with rabbit anti-pig Ig antibodies (Dakopatt, Denmark) bound to solid-phase polystyrene balls (Plastic Ball Co., Chicago, IL) or plates (Immulon, Fisher Sci., USA). After washing, the adsorbed pig Ig can be detected by the same anti-pig antibodies that have been enzyme linked (ie. with peroxidase). Specific isotype of the monoclonal antibodies can be determined by the use of specific anti-pig isotype reagents available commercially (Dakopatt, Denmark).

Once anti-TNF-α (cachectin) monoclonal antibody producing hybridomas have been isolated, they can be further characterized as to binding affinity and epitope specificity.

What we claim is:

1. A polyclonal porcine anti-serum containing polyclonal antibodies capable of binding to human TNF-α (cachectin).

2. A method for preparing antibodies reactive to human TNF-α (cachectin) comprising attaching human TNF-α (cachectin) to a suitable carrier molecule, emulsifying the TNF-α (cachectin) and suitable carrier in an appropriate adjuvant, injecting the TNF-α (cachectin), carrier and adjuvant into pigs, and isolating serum from pigs.

3. A method for preparing antibodies reactive to human TNF-α (cachectin) comprising emulsifying TNF-α (cachectin) in an appropriate adjuvant, injecting the TNF-α (cachectin) and adjuvant into pigs, and isolating serum from pigs.

4. A polyclonal anti-serum produced by the method of claim 2.

5. A polyclonal anti-serum produced by the method of claim 3.

6. A polyclonal anti-serum containing polyclonal antibodies that inhibits the binding of antibodies of claim 1 with TNF-α (cachectin).

7. A polyclonal anti-serum containing polyclonal antibodies that inhibits the binding of antibodies of claim 4 with TNF-α (cachectin).

8. A polyclonal anti-serum containing polyclonal antibodies that inhibits the binding of antibodies of claim 5 with TNF-α (cachectin).

9. A monoclonal antibody reactive to TNF-α (cachectin) which inhibits the binding of antibodies of Claim 1.

10. A monoclonal antibody reactive to TNF-α (cachectin) which inhibits the binding of antibodies of Claim 4.

11. A method for inhibiting the activity of TNF-α on cells comprising the steps of administering to cells an effective amount of anti-TNF-α antibodies as in Claim 1.

12. A method for inhibiting the activity of TNF-α on cells comprising the steps of administering to cells an effective amount of anti-TNF-α antibodies as in Claim 4.

13. A method for inhibiting the activity of TNF-α on cells comprising the steps of administering to cells an effective amount of anti-TNF-α antibodies as in Claim 5.

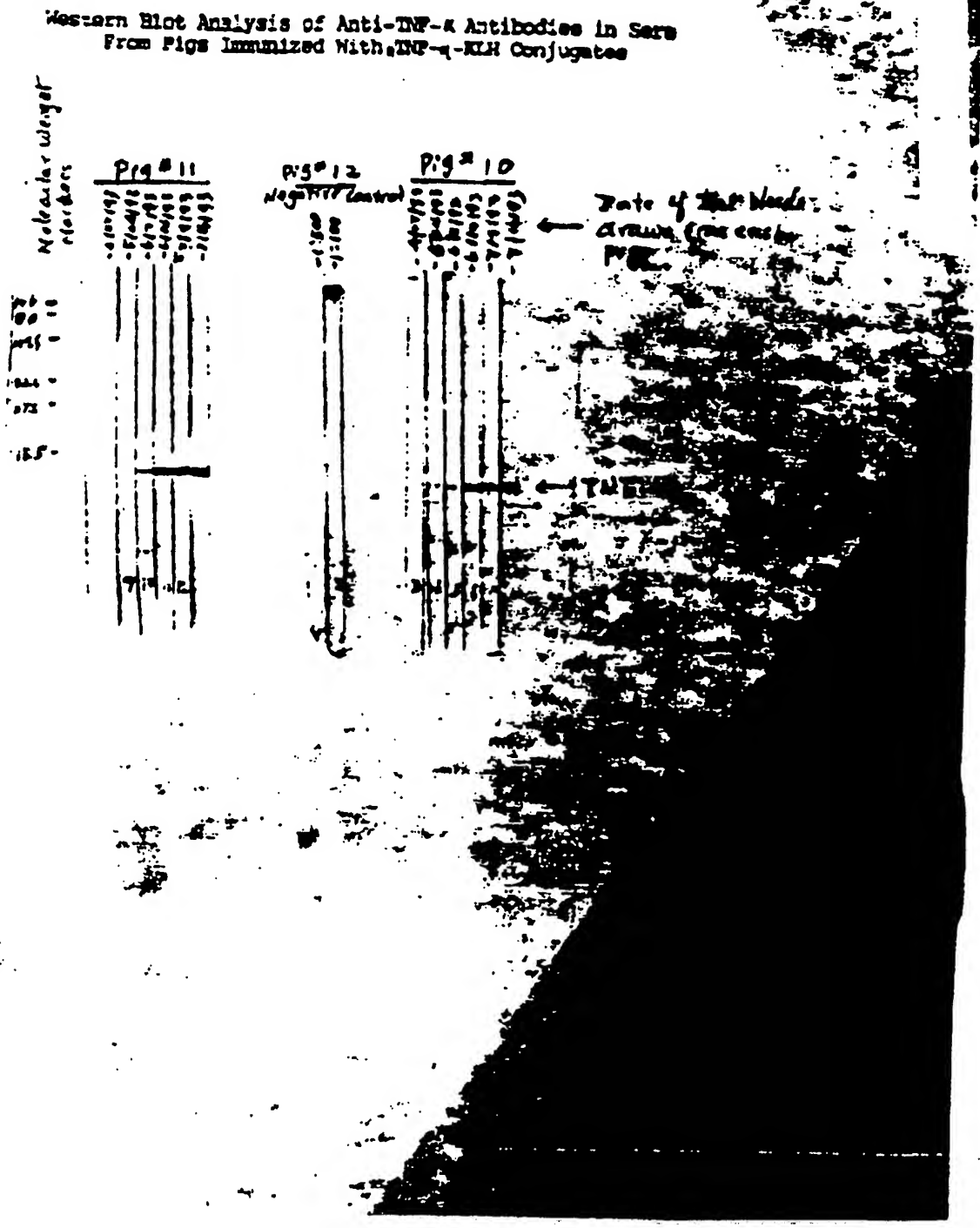
14. A method for inhibiting the activity of TNF-α on cells comprising the steps of administering to cells an effective amount of anti-TNF-α antibodies as in Claim 9.

15. A method for detecting the inhibition of activity of TNF-α comprising the steps of administering to cells an effective amount of anti-TNF-α antibodies, assaying for cell lysis.

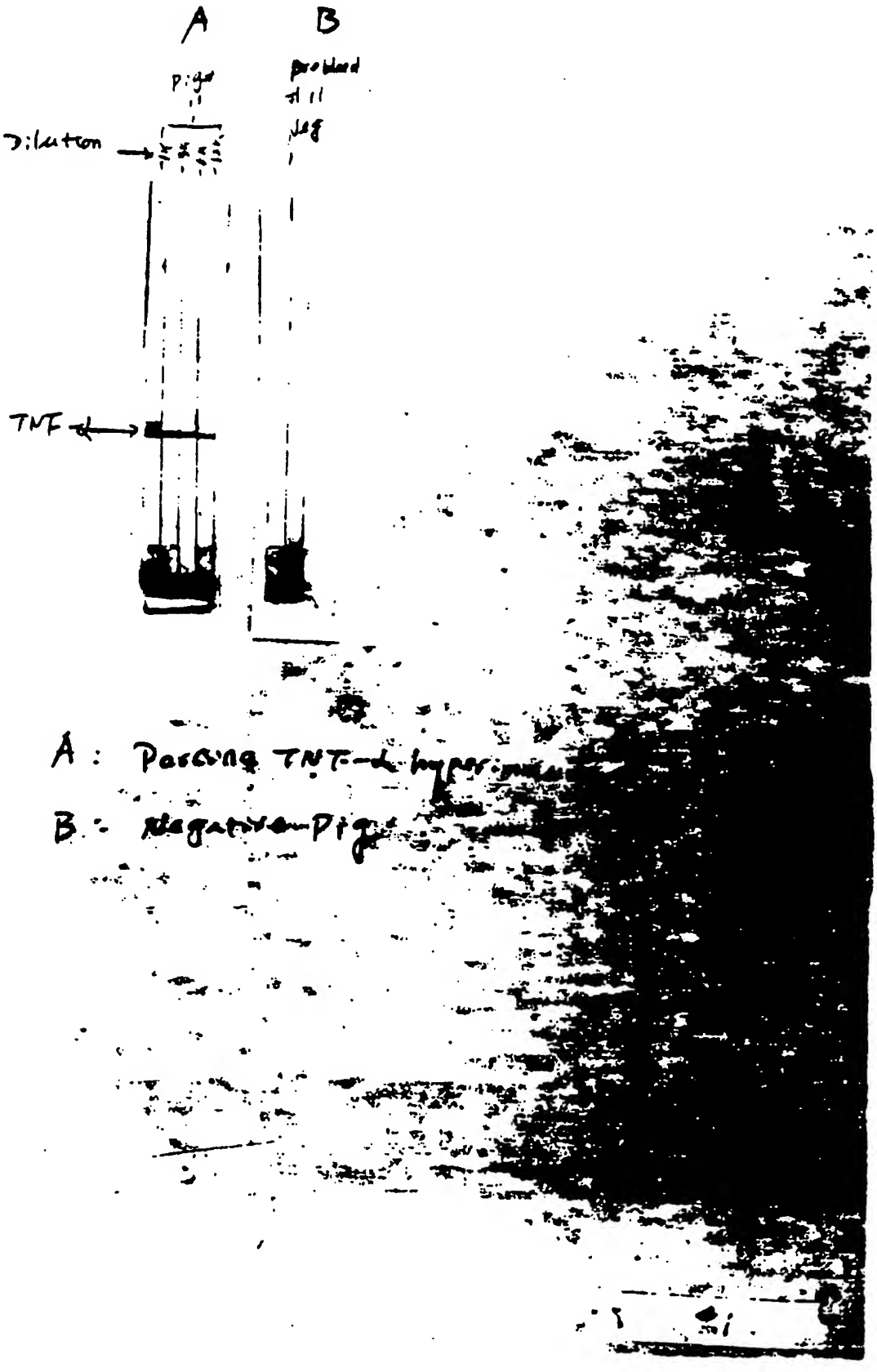
16. A method for detecting the inhibition of activity of TNF-α comprising the steps of administering to cells an effective amount of anti-TNF-α antibodies of Claim 1, assaying for cell lysis.

17.A method for detecting the inhibition of activity of TNF-α comprising the steps of administering to cells an effective amount of anti-TNF-α antibodies of Claim 9, assaying for cell lysis.

Figure 1a


Western Blot Analysis of Anti-TNF-α Antibodies in Sera
From Pigs Immunized With TNF-α-KLH Conjugates
Molecular weight markers
Pig # 11
Pig # 12
Pig # 10
Date of Test Bleeds
TNF

16. Western Blot Analysis of Porcine TNF-α Hyperimmune sera



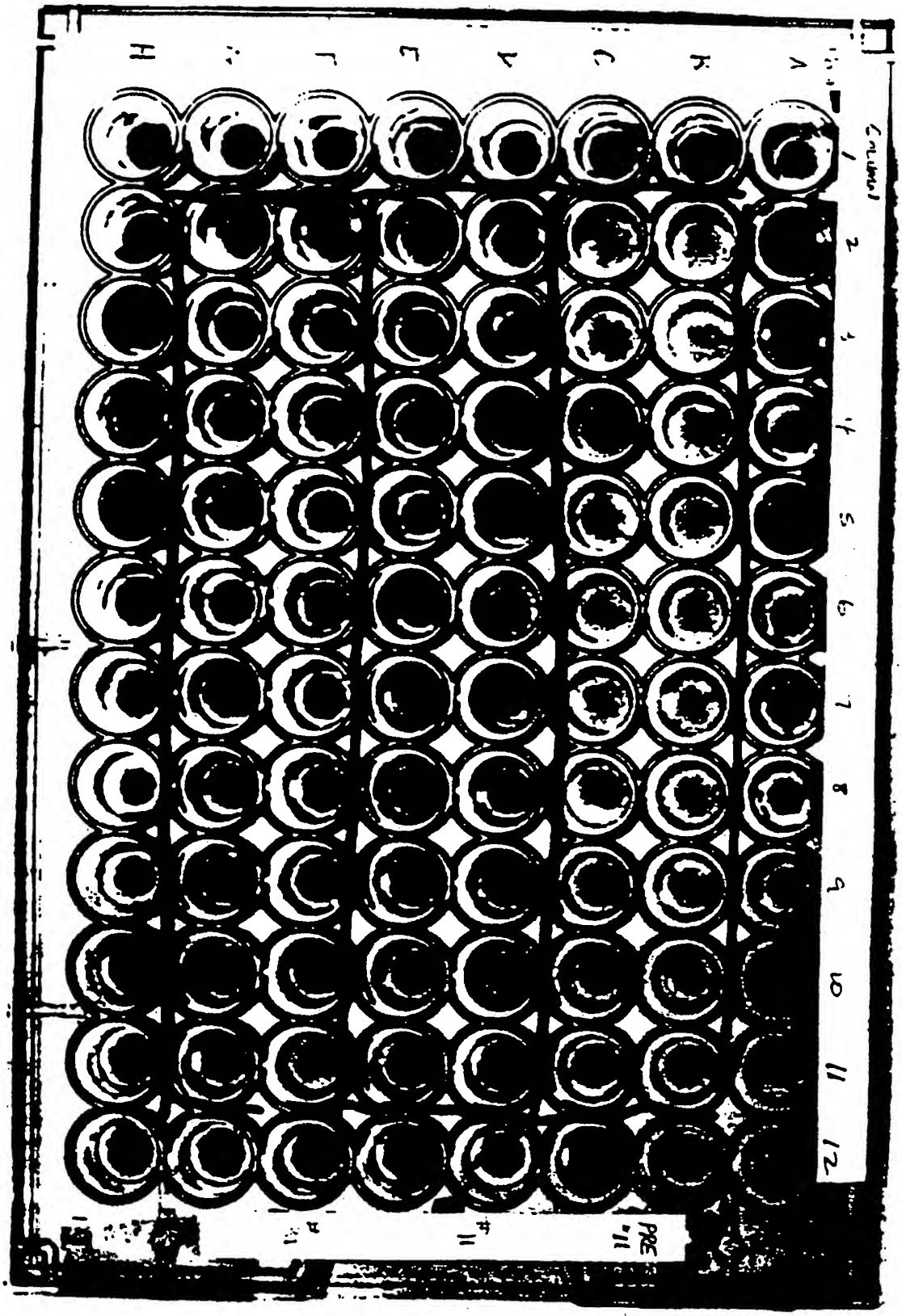


Fig 2

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 95/11700

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/24 C12P21/08 A61K39/395 G01N33/68 G01N33/577
//(C12P21/08,C12R1:91)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12P A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,90 01066 (BIO-RESEARCH LABORATORIES, INC.) 8 February 1990 see page 2, line 23 - page 3, line 14; claims 1,3,5,7,13,15-17,26; example 2 see page 5, line 5 - page 7, line 16	1-5,9-14 15-17
Y	EP,A,0 351 789 (CHIRON CORPORATION) 24 January 1990 see page 7, line 11 - line 36	15-17

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 January 1996	29. 02. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intl. tional application No.
PCT/US95/11700

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 11-14 (as far as being related to an in vivo method) are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/ composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 95/11700

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9001066	08-02-90	EP-A-	0429484	05-06-91
		JP-T-	4500003	09-01-92
EP-A-351789	24-01-90	AU-B-	626572	06-08-92
		AU-B-	3970389	19-02-90
		FI-A-	954230	08-09-95
		IL-A-	90990	21-10-94
		JP-T-	3501330	28-03-91
		NO-A-	952509	22-06-95
		WO-A-	9000902	08-02-90